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PLANT MICROBE INTERACTIONS

Effects of *Agaricus lilaceps* Fairy Rings on Soil Aggregation and Microbial Community Structure in Relation to Growth Stimulation of Western Wheatgrass (*Pascopyrum smithii*) in Eastern Montana Rangeland

The Can Caesar-TonThat · Erin Espeland · Anthony J. Caesar · Upendra M. Sainju · Robert T. Lartey · John F. Gaskin

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Abstract Stimulation of plant productivity caused by *Agaricus* fairy rings has been reported, but little is known about the effects of these fungi on soil aggregation and the microbial community structure, particularly the communities that can bind soil particles. We studied three concentric zones of *Agaricus lilaceps* fairy rings in Eastern Montana that stimulate western wheatgrass (*Pascopyrum smithii*): outside the ring (OUT), inside the ring (IN), and stimulated zone adjacent to the fungal fruiting bodies (SZ) to determine (1) soil aggregate proportion and stability, (2) the microbial community composition and the *N*-acetyl-β-D-glucosaminidase activity associated with bulk soil at 0–15 cm depth, (3) the predominant culturable bacterial communities that can bind to soil adhering to wheatgrass roots, and (4) the stimulation of wheatgrass production. In bulk soil, macroaggregates (4.75–2.00 and 2.00–0.25 mm) and aggregate stability increased in SZ compared to IN and OUT. The high ratio of fungal to bacteria (fatty acid methyl ester) and *N*-acetyl-β-D-glucosaminidase activity in SZ compared to IN and OUT suggest high fungal biomass. A soil sedimentation assay performed on the predominant isolates from root-adhering soil indicated more soil-binding bacteria in SZ than IN and OUT; *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* isolates predominated in SZ, whereas *Bacillus* spp. isolates predominated in IN and OUT. This study suggests that growth stimulation of wheatgrass in *A. lilaceps* fairy rings may be attributed to the activity of the fungus by enhancing soil aggregation of bulk soil at 0–15 cm depth and influencing the amount and functionality of specific predominant microbial communities in the wheatgrass root-adhering soil.

T. C. Caesar-TonThat (✉) · E. Espeland · A. J. Caesar · U. M. Sainju · R. T. Lartey · J. F. Gaskin
United States Department of Agriculture, Agricultural Research Service, 1500 North Central Ave., Sidney, MT 59270, USA
e-mail: thecan.caesar@ars.usda.gov

Introduction

Fairy rings are symptoms of concentric growth of saprophytic soil basidiomycete fungi with the eventual appearance of fungal fruiting bodies. The fungus lives on plant residues at the soil surface [19] and gradually moves outward on an annual basis, fairly uniformly in all directions from the center. Three types of rings are defined based on the effects produced on the herbaceous vegetation: In type I, vegetation is killed; in type II, vegetative production is stimulated; and in type III, there is no effect on vegetation [5, 53]. Fairy rings of type II are reported to stimulate the growth of annual plants, such as *Plantago purshii*, *Festuca octoflora*, *Hedoema nana*, and perennial plants such as *Gutierrezia sarothrae* and *Artemisia frigida* [15, 53, 61] in the area adjacent to the fungal fruiting bodies; however, to our knowledge there is no information on the stimulation of western wheatgrass [*Pascopyrum smithii* (Gaertn.) Á. Löve] by fairy rings.

Nutrient accumulation (mainly N and P) by the fairy ring fungus has been investigated to explain plant growth stimulation (hereafter referred to as the stimulation zone) just inside the zone where fruiting bodies appear in fairy rings. Several authors [15, 18, 38, 53] have demonstrated that fairy ring fungi are capable of mobilizing N and P from organic

matter. For example, Fisher [18] showed that N and P were mineralized during the extension of the fairy ring fungus *Marasmius oreades*, thus resulting in grass growth stimulation. In contrast, in an investigation to evaluate the responses of *Bromus erectus*, *Cynosurus echinatus*, and *Centaurea ambigua* to fairy rings caused by *Agaricus campestris*, Bonanomi et al. [7] reported a depletion of nutrients and no release of inorganic N in the stimulated zone early in the season. They suggested that plant growth enhancement in the stimulation zone could be attributed to changes in microbial community functionality or the promotion of selected species of microorganisms.

Knowledge of soil quality, in particular soil aggregation, of the stimulated zone in fairy rings caused by *Agaricus* is missing. Here, we investigate soil aggregate formation and stability and the changes in the size, composition, and activity of soil microbial communities as affected by *Agaricus lilaceps* fairy rings. Induced shifts are an important component to understand the role of soil aggregation in promoting the productivity of western wheatgrass in Eastern Montana grasslands. Soil aggregates are important for maintaining soil porosity and aeration favorable for plant and microbial growth, infiltration of water, and stability against erosion of soils [60]. Soil aggregate stability is influenced by labile, readily decomposable organic bonding compounds from the organic matter in soil and by microorganisms, such as fungi that can physically entangle soil particles [14] or fungi and bacteria that can produce soil binding agents [11, 28, 64]. As soil organic matter decomposes, organo-mineral associations (e.g., clay humic acids) are formed, allowing structural stabilization. Our hypothesis is that aggregate stability would be greater in the stimulation zone of western wheatgrass in *A. lilaceps* fairy rings, and it would depend on the dynamics of the fairy ring fungus activity.

Western wheatgrass produces nutritious forage, palatable to all classes of livestock during the early growing season, and the species has several characteristics that make it exceedingly valuable for use in revegetation and erosion control: hardiness, drought resistance, and capacity to spread by underground rhizomes [45]. Western wheatgrass has a deep root system and can alleviate soil compaction problems by establishing root channels that increase water infiltration [39], which benefit plant biomass [50]. To better understand some of the fundamental factors that influence the biological mechanisms involved in growth stimulation of western wheatgrass associated with fairy rings, we chose to study the microbial communities in wheatgrass root-adhering soil. Root-adhering soil represents the immediate environment where water and nutrients are taken up by plants, and root exudates have long been considered to represent the major sources of C supporting the growth of root-colonizing bacteria [8]. However, if the plant plays an important role on microbial populations associated with its root system, the influence of soil cannot be excluded. In view of the presumptive evidence that the fairy ring fungus influences soil quality (soil aggregate formation and stability), we investigated the predominant microbial communities that have the potential function to bind [apparent production of binding agents such as extracellular polymer substances (EPS) or presence of cell surface adhesive extensions] to wheatgrass root-adhering soil to ascertain if soil quality could influence the composition of these specific communities.

Three different concentric zones of rings caused by the fungus *A. lilaceps* [outside the circle of fruiting bodies (OUT), just inside the circle of fruiting bodies (stimulated zone or SZ), and inside the stimulated zone toward the center of the ring (IN)] were compared for some key factors of soil quality. The objectives of this study were (1) to determine soil aggregation at the soil depth of 0–15 cm by measuring the proportion of different aggregate-size classes (4.75–2.00, 2.00–0.25, and <0.25 mm) and their mean-weight diameter; (2) to investigate whether any changes in soil aggregation correlated with changes in the broad microbial community structure and with *N*-acetyl-β-D-glucosaminidase activity in bulk soil at the soil depth of 0–15 cm; (3) to assess the proportion of the predominant culturable bacterial communities in soil adhering to roots of western wheatgrass, in particular bacteria that have the potential to bind soil; and 4) to quantify the stimulation of western wheatgrass production by *A. lilaceps* fairy rings.

Materials and Methods

Site Description

The experiment was conducted in grassland area northwest of Sidney (47°42′ N and 104°28′ W, elevation 738 m), Montana USA, in 2010 and 2011. The main area is characterized by wide variations in mean monthly air temperature from −8 °C in January to 23 °C in July and August. The mean annual precipitation (105 years average) is 340 mm, 80 % of which occurs from April to October. Within an area of approximately 10 km^2 where many fairy rings caused by various basidiomycete fungi could be found, three different fairy rings of *A. lilaceps* were selected. Molecular identification of the fungus is described below. The *A. lilaceps* fairy ring locations were chosen because they were similar in size (about 200 m in diameter) and because they all appeared to stimulate western wheatgrass. Location 1 (749.4 m altitude) was south facing and about 2.0 km from location 2 (753.2 m altitude) and location 3 (760.8 m altitude), which were north facing; locations 2 and 3 were about 1.0 km apart. Table 1 indicates the soil properties at fairy ring locations at an average depth profile of 0–15 cm. Organic matter content and the concentrations of ammonium (NH_4–N), nitrates (NO_3–N), and phosphorus (P) of soil collected at the depth

Table 1 Soil properties in various grassland locations with *Agaricus lilaceps* fairy rings in Eastern Montana at an average depth profile of 0–15 cm

Soil properties	Location		
	Location 1	Location 2	Location 3
Sand (g kg^{-1})	520	520	500
Silt (g kg^{-1})	320	380	410
Clay (g kg^{-1})	150	90	80
pH	6.07	5.97	6.07

Dominant grasses are western wheatgrass [*Pascopyrum smithii* (Gaertn.) A. Love], crested wheatgrass [*Agropyron cristatum* (L.) Gaertn.], and green needle grass [*Nassella viridula* (Trin.) Backworth]

of 0–15 cm were analyzed by A & L Western Agricultural Laboratories, Modesto, CA, USA using the standard methods of WREP 125 [65] (Table 2). Grazing management was implemented in early 1960 and was typically 94 cow/calf pairs for about 240 ha during the months of June through August. Wild antelope also graze at this site with herds varying from 15 to 20 animals some years and 40–50 animals per herd in others.

Vegetation and Soil Sampling

Plant and soil samples from three *A. lilaceps* fairy rings were collected in June–July 2010 and 2011 when the fungus was producing fruiting bodies. At each ring, soil and vegetation were collected from (1) SZ, 40–50 cm adjacent to the fungal fruiting bodies toward the center of the ring; (2) IN, 1 m from the stimulated zone toward the center of the ring; and (3) OUT, 1 m from the fungal fruiting bodies outside the ring. Within each zone, three 20×50 cm quadrats spaced 20–30 m apart were used to record vegetation data. The heights of 30 western wheatgrass plants inside each quadrat were recorded. Plants were then removed with their root material and placed in a cooler for transport to the laboratory where roots were processed for isolation of bacteria. In addition to plant materials, a hand probe (5 cm inside diameter) was used to collect six soil cores per quadrat at 0–15 cm soil depth, after removing the surface plant residues. Soil from the cores was immediately refrigerated, bulked, and homogenized by mixing and removing roots and coarse organic materials, and then was processed in the laboratory for soil aggregate fractionation and stability, fatty acid methyl esters (FAMEs) extraction and analysis, and determination of *N*-acetyl-β-D-glucosaminidase activity. Bulk soil from the cores was also used to determine soil organic matter content and concentration of NH_4–N, NO_3–N, and P. The percent cover of western wheatgrass was visually estimated. Three subsamples of cover were taken using a 20 cm×50 cm quadrat near each soil sampling location in each year. In 2011, demographic data on western wheatgrass were collected: the number of stems (or tillers) within the quadrat and the number of inflorescences. Aboveground biomass of western wheatgrass was clipped within each quadrat and the biomass dried at 65 °C for 3 days before weighing.

Table 2 Soil organic matter content and concentration of ammonium (NH_4–N), nitrate (NO_3–N), and phosphorus (P) at 0–15 cm soil depth from different sampling zones at different locations of fairy ring caused by *Agaricus lilaceps*

	IN	SZ	OUT
Organic matter (%)	3.7a	5.5a	4.1a
NH_4-N (mg kg^{-1})	12.00b	23.5a	14.00ab
NO_3-N (mg kg^{-1})	21.33b	34.50a	18.00b
P (mg kg^{-1})	11.67a	18.67a	14.00a

Values which share the same letters within a row are not significantly different at $P \leq 0.05$ by honestly significant difference procedure of Tukey and Kramer

IN inside the ring, *SZ* stimulated zone, *OUT* outside the ring

Soil Aggregate Fractionation

Aggregates from 2010 to 2011 soil samples were separated by dry sieving of moist soil as described by Mendes et al. [41]. Field-moist soil was air-dried at 4 °C for 3–7 days until reaching gravimetric water content of 100 g kg^{-1}. This water content represented the moisture level at which soils can be sieved in finer sieves for aggregate-size separation. Drying at 4 °C reduces impact on microbial communities and activity in aggregates [41]. Dry sieving of aggregate separation was used in preference to wet sieving because dry sieving may reduce the disruption of the physical habitat of microbial communities in aggregates compared to wet sieving [41] and aggregates separated by dry sieving may represent more closely those in the field in the absence of rain; the method is more appropriate for Eastern Montana rangeland where soil erosion is greater due to the action of wind. The aggregate proportion in soil (gram aggregate per kilogram soil) was measured in aggregate-size classes of 4.75–2.00, 2.00–0.25, and <0.25 mm. Mean weight diameter (MWD), defined as the sum of the weighted mean diameters of all size classes and used as an index of aggregate stability was calculated according to the procedure described by Kemper and Rosenau [32].

Isolation of Root-Adhering Soil Bacteria

One gram of fresh western wheatgrass roots (0.5–1 cm diameter) with adhering soil was soaked in sterile $MgSO_4$ buffer (0.1 M, pH7.3) with glass beads (0.50 mm) for 16 h at 4 °C on a rotary shaker. Soil suspensions were transferred

to microporous filter-equipped plastic bags (Seward Medical, London, UK) and homogenized to separate the bacteria from larger particles. Suspensions were placed on low nutrient medium [12, 49] using a spiral plater (Don Whitley Scientific Limited, West Yorkshire, UK). The basic concept of spiral plating is to continuously deposit a known volume of sample on a rotary agar plate in the form of an Archimedes spiral [22, 30]. The amount of sample evenly decreases while the dispensing stylus is moved from the center to the edge of the rotating agar plate. From the beginning to the end of the spiral at the periphery of the plate represents a three logarithmic unit dilution when 150 μL aliquots of soil suspensions were plated [30], thus 10^5 cells g^{-1} soil represented a typical population level at which the colonies considered the predominant isolates were obtained. Colonies growing at the end of the spiral plates were collected; a total of 72 colonies represented the predominant bacteria for each concentric zone of each ring. Thus, in total, 648 colonies from soil samples collected in the three sampling zones of the fairy rings from the three locations were isolated. The colonies were further purified and stored at −80 °C in Luria–Bertani medium amended with 15 % glycerol. All isolates were identified individually to species using fatty acid methyl ester (FAME) profiling as described below. Then, isolates were individually assayed (soil sedimentation assay) to determine their potential to aggregate soil. Species that can function as soil aggregators were further DNA sequenced for additional identification. Only samples from 2011 were used to isolate root-adhering soil bacteria.

Soil Sedimentation Assay

The soil sedimentation assay was used to screen the root-adhering soil bacterial species for their capability to bind soil particles following the procedure of Caesar-TonThat et al. [12]. Briefly, triplicated isolates (10^6 cells ml^{-1}) from pure cultures were washed with deionized water [36] then transferred to glass tubes containing deionized water and soil (14 % clay, 14 % silt, and 72 % sand) that was previously sieved to a size of <0.05 mm. A control without bacteria was prepared in a manner similar to each individual isolate. Tubes were vortexed for 10 s at 2,250 rpm, and the mixture was allowed to settle for 5 min. Images of the reflected light [Universal/Hi-vision fluorescent light F32T8/TL735, Philips, NY, USA, light intensity 7.435± 0.064 Rad (watt/m^2)] for multiple samples were captured using a digital camera (Nikon, model D-80, Japan) with night vision settings (near infrared, 800–1,000 nm) at 24.78 °C±0.746. The captured images were calibrated by referring white (255 in grayscale value) and black (0 in grayscale value) image spots. Adobe Photoshop (version 7.0) was used for the conversion of the images in a grayscale value of each target solution into the reflectance (expressed in percent) that directly correlates to relative differences of the solution density: 100 % reflectance for maximum density and 0 % for minimum density. A reflectance ratio (percent reflectance of the solution with bacterial cells added divided by the reflectance of control solution without cells added) was calculated for each isolate, and values were averaged for all the isolates for each species. The solution with reflectance ratio>2 was established as a threshold, which represents species that were the most efficient to bind soil. Ratios<2 represented the activities of species that were inconsistent or marginal in soil binding activity.

FAME Profiling and DNA Sequencing of Bacterial Isolates

Two separate FAME extraction methods were used in this study. A first method followed the MIDI protocol (Microbial Identification System, Inc., Newark, NJ, USA) to identify individual bacterial isolates from soil adhering to wheatgrass roots. A second extraction method followed the protocol described by Buyer et al. [9] to analyze specific fatty acids in bulk soil that have been proposed as biomarkers to broad taxonomic microbial groups. In the latter extraction method, bulk soil samples were run through the gas chromatography column for 4 min, long enough for fatty acids up to 28 carbons long to pass through. Individual bacterial FAMEs and whole soil FAMEs were analyzed by gas liquid chromatography (Agilent 6,890N, Agilent Technologies Inc., Wilmington, DE, USA). Fatty acids were identified on the basis of their retention times relative to known standards and quantified. Fatty acids of individual bacteria were identified following the MIDI eukaryotic method. Species names with SIM>0.500 were considered a good match [46]. Specific FAMEs in whole soil were identified and quantified following the MIDI PLFAD1 method [10].

Specific individual fatty acids extracted from bulk soil were used to calculated the mean fatty acid ratio of fungi to bacteria (the sum of mole percent for 18:2ω6*c*, 18:1ω9*c*, and 18:1ω9*t*, to that for *i*15:0, *i*16:0, *a*15:0, 14:0, 15:0, 16:1ω7*c*, 16:1ω5*c,* *a*17:0, *i*17:0, 17:0, and 18:0) [3, 9, 17, 20, 63]. The designation of fatty acids follows the convention *X*:*Y*ω*Z* where "*X*" is the total number of carbon atoms in the molecule, "*Y*" the number of double bonds, "*Z*" the position of the first double bond or cyclopropane ring, and "ω" the position counted from the methyl end of the molecule. The prefix "*i*" refers to *iso* branching and "*a*" to *anteiso* branching; "*c*" and "*t*" refer to *cis* and *trans* configuration. In this study, samples from 2010 to 2011 were performed in triplicate.

To corroborate identification made by FAME profiling with molecular-based methods of identification, the most efficient soil binding species identified by FAME [species with a reflectance ratio>2 and highest similarity index (SIM)] were further processed for DNA sequencing to confirm their identity at the genus level. DNA from the isolates was extracted using a Qiagen (Valencia, CA, USA) DNeasy Tissue kit. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene region used primers16S-27f (5′-GAGTTTGATCCTGGCTCAG-3′) and 16S-960r (5′-

GCTTGTGCGGGYCCCCG-3′) with the following cycling conditions: 95 °C (10 min); 25 cycles of 94 °C (30 s), 56 °C (30 s), 72 °C (2 min); and then 72 °C (2 min). A 50 μl reaction was performed for each isolate, and PCR products were purified using a QIAquick PCR Purification kit (Qiagen). Purified templates were sequenced in two directions with an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA), using the same primers listed above. The DNA sequences have been deposited in GenBank and the accession numbers are indicated in Table 5.

Molecular Identification of *Agaricus lilaceps*

Pure cultures of the fairy ring fungal isolate FR1 were obtained by isolation of basidiocarp tissue on potato dextrose agar similar to the methods of described in Stamets and Chilton [56]. Extract-N-Amp Plant PCR Kits (Sigma Chemical Co., St. Louis, MO, USA) were used to prepare the fungal DNA templates for the PCR according to the protocol of Lartey et al. [34]. The 20 μl PCR reaction consisted of 10 μl Extract-N-Amp PCR mix (a 2× PCR reaction mix containing buffer, salts, dNTPs, Taq polymerase and TaqStart antibody), 4 μl sample extraction solution, 1.5 μl each of the ITS1 forward and ITS4 reverse ITS primers [66], in deionized water. Amplification was carried out over 35 cycles using a Mastercycler gradient thermocycler (Eppendorf Scientific Inc., Westbury, NY, USA) at 94 °C for 1 min denaturation, 52 °C for 30 s annealing, and 72 °C for 1 min extension. The PCR amplified products were resolved by electrophoresis in 1 % agarose gels. Amplicons were carefully excised from the agarose gel, weighed, and purified with QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) with slight alteration of the manufacturer's protocol [34]. The purified DNA was stored at −20 °C prior to sequencing. The purified amplicon DNA fragments were sequenced as described by Lartey et al. [34] using BigDye® Cycle Terminator Sequencing v 3.1 kit (Applied Biosystems, Inc., Foster City, CA, USA) The purified amplicons from the gels was sequenced four times. The sequences were imported into the Vector NTI Advance v.11 sequence and data management suite (Invitrogen Corp., Carlsbad, CA, USA), to generate a consensus sequence for the *Agaricus*. The sequence has been deposited in GenBank and can be accessed under the number JX129888. After a BLAST search, related sequences were retrieved from GenBank. These were compared by multiple alignment using ClustalW2 method, and a neighbor-joining phylogenetic tree using Kimura 2-parameter matrix with 1,000 bootstrap replicates was constructed in MEGA 5.10 software [59] (Fig. 1).

N-Acetyl-β-D-glucosaminidase Activity

N-Acetyl-β-D-glucosaminidase activity has been positively correlated with both fungal biomass and C and N mineralized in soil [1, 47], in particular with the decomposers of lignocellulosic substrates in soil [43, 51]. The activity of *N*-acetyl-β-D-glucosaminidase was determined according to the method of Parham and Deng [47]. Briefly, soil (1 g) was mixed with acetate buffer (0.1 M, pH5.5) and ρ-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma, St Louis, MO, USA) solution (10 mM) used as substrate. The mixture was incubated for 1 h at 37 °C. After incubation, $CaCl_2$ (0.5 M) and NaOH (0.5 M) were added to the mixture to stop the reaction. Samples were filtered through Whatman no. 2 filter paper. The color intensity of the filtrate was measured at 405 nm with a spectrophotometer. Soil samples collected in 2010 and 2011 were assayed in triplicate. Controls with the substrate being added after the reactions were stopped, and controls without addition of soil to the reaction mixtures were performed. The activity of *N*-acetyl-β-D-glucosaminidase was expressed in milligrams ρ-nitrophenol released per kilogram soil per hour. The ρ-nitrophenol content was calculated using a standard curve for ρ-nitrophenol as described by Tabatabai and Bremner [58].

Statistical Analysis

The honestly significant difference (HSD) test of Tukey–Kramer [57] in ANOVA procedure was used to analyze data of 2010 and 2011 on soil aggregate distribution, microbial functional groups, proportion of fatty acids, and enzymatic activity. The fixed effects in the analysis were years, location, and sampling zones, whereas random effects were replication. Means were separated using the least square means test when the interaction was significant. Difference in significance level was evaluated at $P \leq 0.05$ using the JMP statistical software package (version 9.0). Percent cover was square root transformed for analysis to improve the distribution of residuals. In 2010, cover in a generalized linear model using zone, location and the zone×location interaction as factors was examined. When a factor was significant, Tukey and Kramer's HSD was used to test for differences among means. Our a priori hypothesis in 2011 was that western wheatgrass performance (number of stems, number of inflorescences, height, and biomass) would be affected by location, zone, and/or their interaction, so univariate analyses using generalized linear models on least square means were performed on each western wheatgrass performance variable. Principal components analysis (PCA) was used to show relationships among the different concentric zones (IN, SZ, and OUT) of the fairy rings based on the correlations of multiple variables [organic matter content, NH_4–N, NO_3–N, and P concentrations, aggregate stability (MWD), FAME ratio of fungi/bacteria, Gram-positive (GP) and Gram-negative (GN) bacteria, β-glucosaminidase activity, wheatgrass cover] from each zone. Principal components analysis was performed on data values from 2010 to 2011, using JMP software (version 9.0).

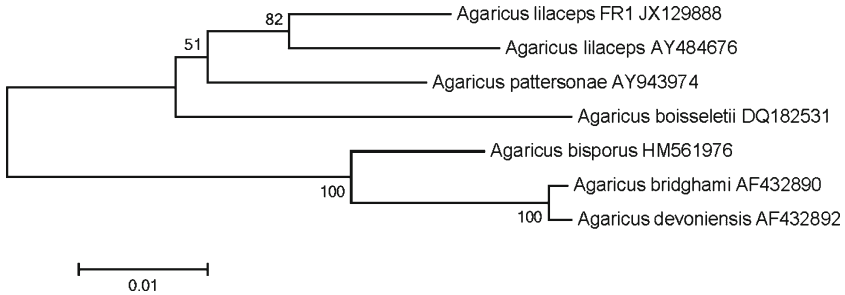

Fig. 1 Neighbor-joining phylogram based on alignment of ITS sequence of the fairy ring fungus *Agaricus lilaceps* FR1 (GenBank accession number JX129888) isolated from eastern Montana with sequences of other *Agaricus* spp. from GenBank. *A. lilaceps* FR1 clustered with AY484676 from GenBank. The scale on the bottom of the phylogram indicates a genetic distance of 0.01

Results

The target ITS region of the fairy ring fungus (isolate FR1) was successfully amplified using ITS1 and ITS4 primers, purified, and sequenced. The sequence from *A. lilaceps* FR1 clustered together with *A. lilaceps* (GenBank accession number AY484676). The sequence separated from other *Agaricus* species from GenBank including *Agaricus bisporus* HM561976, *Agaricus bridghami* AF432890, *Agaricus devoniensis* AF432892, *Agaricus boisseletii* DQ182531, and *Agaricus pattersonae* AY943974 (Fig. 1).

Soil concentration for NH_4–N and NO_3–N were higher in SZ than in IN and OUT (Table 2). The content of organic matter and the concentration of P did not differ significantly in soil of the three concentric sampling zones.

Table 3 indicates that soil aggregate distribution by dry-sieving varied with sampling zones (SZ, IN, and OUT) at 0–15 cm soil depth. The amount (grams per kilogram soil) of large macroaggregates (4.75–2.00 mm) and small macroaggregates (2.00–0.25 mm) was higher in SZ than IN and OUT in all three rings in both years. Mean proportion showed 1.7 times more large macroaggregates in SZ than in IN, 1.4 times more than OUT, but no significant difference between IN and OUT. Similar mean proportion results were found for the small macroaggregates with 1.2 and 1.3 times more in SZ than IN and OUT, respectively, but with no difference between IN and OUT. This resulted in smaller proportions of microaggregates (< 0.25 mm) in SZ compared to IN and OUT. In both years, mean weight diameter (MWD) was highest in SZ compared to IN and OUT zones of the three rings, but there was no significant difference between IN and OUT. In general, the proportion of large macroaggregates and aggregate stability was higher in 2011 than in 2010.

FAME profiles of soil microbial communities of bulk soil collected at 0–15 cm depth at the concentric sampling zones are indicated in Table 4. Bulk soil in SZ contained significantly higher FAME ratio fungi/bacteria than IN and OUT, but no difference was found between IN and OUT. Measurement of the activity of *N*-acetyl-β-D-glucosaminidase indicated that the mean value in SZ was 1.75 times higher than IN and about 1.50 times higher than OUT. *N*-Acetyl-β-D-glucosaminidase activity between IN and OUT was not significantly different.

Table 3 Aggregate proportion and mean-weight diameter (MWD) from bulk soil collected at 0–15 cm depth at the plant stimulated zone (SZ), inner zone (IN), and outer zone (OUT) of fairy rings caused by *Agaricus lilaceps*

Year	Fairy ring	Sampling zone	Aggregate proportion in size class (g kg^{-1} soil)			Mean-weight diameter (mm)
			4.75–2.00 mm	2.00–0.25 mm	0.25–0.00 mm	
2010			178.7b[a]	584.6a	236.7a	1.29a
2011			216.3a	590.9a	192.8a	1.42a
	Location 1		230.3a	612.4a	157.4b	1.49a
	Location 2		191.8ab	590.8ab	217.4ab	1.34ab
	Location 3		170.4b	559.9b	269.6a	1.24b
		SZ	257.3a	632.7a	110.0b	1.61a
		IN	150.1b	572.9b	277.0a	1.19b
		OUT	185.1b	557.7b	257.3a	1.29b

[a] Within a set in a column, numbers followed by different letters are significantly different by the Honestly Significant Difference procedure of Tukey and Kramer at $P \leq 0.05$

Table 4 Mean fatty acid methyl ester values of Fungi/Bacteria ratios and N-acetyl-β-D-glucosaminidase activity in bulk soil collected at 0–15 cm depth at the stimulated zone (SZ), inner zone (IN), and outer zone (OUT) of fairy rings caused by *Agaricus lilaceps*

Year	Sampling location	Sampling zone	Fungi/Bacteria Ratio	N-acetyl-β-D-glucosaminidase Activity
2010			0.58a[a]	44.39a
2011			0.56a	43.16a
	Location 1		0.53a	36.02b
	Location 2		0.56a	50.23a
	Location 3		0.61a	45.05ab
		SZ	0.63a	58.64a
		IN	0.53b	33.49b
		OUT	0.54b	39.18b

Fatty acids are calculated using relative abundances (mole percent of total fatty acids). *N*-Acetyl-β-D-glucosaminidase activity is expressed in milligrams ρ-nitrophenol per kilogram soil per hour

[a] Within a set in a column, numbers followed by different letters are significantly different by the honestly significant difference procedure of Tukey and Kramer at $P \leq 0.05$

The distribution of the culturable and predominant bacteria in western wheatgrass root-adhering soil of fairy rings that were individually identified by FAME profiling showed significantly higher amounts of γ-Proteobacteria (36.56 %), including the pseudomonads (26.15 %), in SZ compared to IN (3.45 and 2.75 %, respectively) and OUT (14.46 and 14.46 %, respectively), but Bacilli-Actinobacteria were more numerous in IN (59.20 %) and OUT (39.10 %) than in SZ (24.14 %) (Fig. 2). In general, Gram-positive bacteria were more numerous in root-adhering soil of IN (91.20 %) and OUT (67.97 %) compared to SZ (50.77 %), whereas Gram-negative bacteria predominated in SZ (49.23 %) compared to IN (8.97 %) and OUT (32.02 %). A soil sedimentation assay was performed on all isolates to determine which ones have the potential to bind soil particles. Western wheatgrass root-adhering soil from SZ had significantly more soil-binding bacteria (26.36 %) than IN (15.14 %), but did not differ from OUT (20.37 %). Table 5 indicates the distribution of bacterial species that can bind soil the most efficiently (reflectance ratio >2) found in root-adhering soil of western wheatgrass in the three concentric zones that were identified by FAME profiling and DNA sequencing. Root-adhering soil in SZ contained the most *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia*, and *Bacillus cereus* was the most abundant in IN and OUT. Identification to genus by DNA sequencing of the soil-aggregating bacteria appeared to corroborate with the identification by FAME profiling, except that sequencing by DNA identified

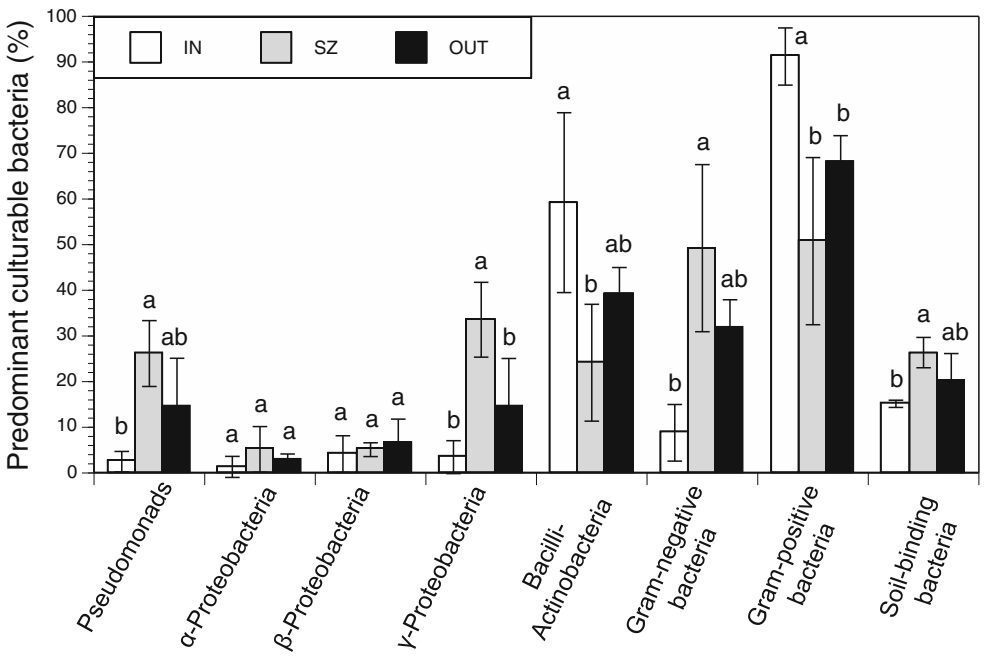

Fig. 2 Percent distribution of the predominant main groups of bacteria and soil-binding bacteria isolated from wheatgrass root-associated soil collected in three concentric zones (*SZ* stimulated zone; *IN* inside the ring; *OUT* outside the ring) of *Agaricus lilaceps* fairy rings in eastern Montana grassland. Bars are standard deviation; different letters indicate significant difference by the honestly significant difference procedure of Tukey and Kramer at $P \leq 0.05$

Table 5 Distribution of the predominant culturable soil-aggregating bacterial isolates from soil adhering to roots of western wheatgrass growing in fairy rings caused by *A. lilaceps*

FAME identification	SZ	IN	OUT	Reflectance ratio[a]	DNA identification[b]			
Species					Family	Genus	Accession number	% SIM
Gram-positive bacteria								
Bacillus atrophaeus (0.861)[c]	4	6	6	4.21	*Bacillaceae*	*Bacillus*	KC196704	98.9
Bacillus cereus (0.714)	2	16	14	4.58	*Bacillaceae*	*Bacillus*	KC196705	99.0
Bacillus sphaericus (0.471)	0	4	1	3.24	*Planococcaceae*	*Lysinibacillus*	KC196706	96.7
Gram-negative bacteria								
Bradyrhizobium japonicum (0.431)	0	0	1	2.55	*Phyllobacteriaceae*	*Phyllobacterium*	KC196707	98.9
Rhizobium radiobacter (0.880)	4	0	0	2.12	*Rhizobiaceae*	*Beijerinck ia*	KC196708	95.4
Rhizobium rubi (0.667)	1	0	0	2.64	*Rhodocyclaceae*	*Shinella*	KC196709	97.6
Chryseobacterium balustinum (0.636)	1	0	1	2.01	*Flavobacteriaceae*	*Chryseobacterium*	KC196710	93.0
Chryseobacterium indoltheticum (0.884)	1	0	1	2.00	*Flavobacteriaceae*	*Chryseobacterium*	KC196711	94.5
Pseudomonas fluorescens biotype B (0.907)	20	0	0	3.54	*Pseudomonadaceae*	*Pseudomonas*	KC196712	95.9
Pseudomonas fluorescens biotype G (0.925)	6	0	0	3.66	*Pseudomonadaceae*	*Pseudomonas*	KC196713	95.4
Pseudomonas syringae (0.683)	3	0	5	2.32	*Pseudomonadaceae*	*Pseudomonas*	KC196714	95.6
Stenotrophomonas maltophilia (0.705)	10	1	2	3.10	*Xanthomonadaceae*	*Stenotrophomonas*	KC196715	95.7
Brevundimonas vesicularis (0.505)	1	2	3	2.06	*Caulobacteriaceae*	*Brevundimonas*	KC196716	97.5
Burkholderia cepacia (0.402)	0	0	1	3.44	*Burk holderiaceae*	*Burkholderia*	KC196717	98.7
Sphingobacterium spiritivorum (0.745)	0	0	2	2.51	NA	NA	NA	NA
Zoogloea ramigera (0.561)	2	0	2	2.01	*Phyllobacteriaceae*	*Phyllobacterium*	KC196718	98.3

NA not available, *SZ* stimulated zone, *IN* inside the ring, *OUT* outside the ring, *SIM* similarity index

[a] Average of reflectance ratio (reflectance measurement of soil suspension containing 10^6 cells/ml after 5 min of sedimentation time divided by relectance measurement of soil suspension without bacteria added) obtained from all isolates of each soil aggregating species

[b] Isolates from species that have the highest similarity index in FAME analysis and the reflectance ratio >2.0 were DNA sequenced and analyzed

[c] Similarity index between parentheses was based on MIDI Aerobic Bacterial Library

Zoogloea and *Bradyrhizobium* as *Phyllobacterium*, and *Rhizobium* as *Shinella*.

There was a significant main effect of zone on western wheatgrass cover in both years (Table 6). Cover of western wheatgrass was higher in SZ compared to IN and OUT (Table 7). In 2011, all measures of western wheatgrass performance were significantly greater in SZ than IN or OUT (Tables 6 and 7). Based on the correlations of multiple variables indicated in Fig. 3b from each zone of the rings, PCA analysis (Fig. 3a) separated the zones into two distinct clusters: SZ zone clustering (circles) was separated from IN (triangles) and OUT (squares) along PC1, which accounted for 43.8 % of the variability; PC2 differentiated location 1 from 2 to 3, which accounted for 18.2 % of the variability.

Discussion

This study demonstrates that the passage through soil of the basidiomycete fungus *A. lilaceps* as fairy rings in Eastern Montana grassland had a clear effect on the biotic and abiotic properties of the soil. The concentric zone adjacent to the fungal fruiting bodies that stimulates western wheatgrass constitutes a dynamic ecological system where the fairy ring fungus was the most active in influencing soil properties and microbial community structure.

When bulk soil in SZ was compared to IN and OUT of the fairy rings, the increase in macroaggregate formation (4.75–2.00 and 2.00–0.25 mm) and aggregate stability in SZ could be partly related to the dynamics of fungal activity. Fungi can act as a web to physically aggregate soil particles, and they can produce mucilage (microbial cellular materials and exopolysaccharides) [11, 28, 64] on the surface of their hyphae that can strongly attach to inorganic materials [6, 54] resulting in aggregate stabilization. There was an increase in the amount of fungi in SZ compared to IN and OUT, as demonstrated by the higher fatty acid fungal:bacterial ratio and glucosaminidase activity. A shift in microbial community composition to higher fungal/bacterial ratios has been associated with potential changes in soil quality and C sequestration in diversified cropping systems and conservation tillage [4]. The higher aggregate stability in 2011 than

Table 6 Data analysis for western wheatgrass: univariate analyses on vegetation data (western wheatgrass stem number, number of inflorescences, height, and biomass not measured in 2011)

		2010		2011	
		F	*P* value	*F*	*P* value
Cover (%)	Location	2.22	0.138	2.75	0.091
	Zone	21.19	*<0.0001*	36.43	*<0.0001*
	Location×zone	1.47	0.252	2.66	0.067
Number of stems	Location			1.89	0.183
	Zone			43.35	*<0.0001*
	Location×zone			0.55	0.703
Number of Inflorescences	Location			2.12	0.149
	Zone			16.21	*<0.0001*
	Location×zone			2.58	*0.072*
Height (cm)	Location			0.41	0.668
	Zone			69.13	*<0.0001*
	Location*zone			1.81	0.172
Biomass (g)	Location			1.04	0.375
	Zone			85.74	*<0.0001*
	Location×zone			1.2	0.349

Significant *P* values (< 0.05) are in italics.

in 2010 was probably caused by higher precipitation in spring–summer 2011 compared to the previous year, resulting in enhanced organic matter decomposition, more microbial activity, and production of soil binding agents by microorganisms.

Terrestrial fungi, in particular basidiomycetes, produce a wide range of enzymes (e.g., hemicellulases, cellulases chitinases, and ligninases) and are therefore able to convert polymeric compounds, such as cellulose, hemicelluloses, and lignin, into smaller molecules that can be assimilated by other microorganisms or by plants [13]. Our finding of an increase in *N*-acetyl-β-D-glucosaminidase enzyme activity in the stimulated zone of the fairy rings compared to the other zones suggests higher fungal biomass in that zone. The enzyme *N*-acetyl-β-D-glucosaminidase is a glycosidase [47] that is implicated in fungal hyphal extension and branching, septa formation, and germ tube growth [1, 2, 51], and its activity appears positively and quantitatively correlated with ergosterol content in environmental samples [33]. In addition, *N*-acetyl-β-D-glucosaminidase activity has been demonstrated to be positively correlated to the cumulative N mineralized in soils [16]. Our finding of higher soil concentrations of NH_4–N and NO_3–N in SZ indicates a possible role of *A. lilaceps* in N mineralization. These data are in accord with other studies of fairy rings caused by *M. oreades* [61], *A. campestris* [7], and *Agaricus arvensis* [15] where high NH_4–N and NO_3–N release were demonstrated in the stimulated zone. Furthermore, Mathur [38] has demonstrated that the fairy ring fungus *M. oreades* is capable of degrading the humus fractions fulvic acids and humic acids in soil. In this context, we speculate that the high NH_4–N and NO_3–N soil concentrations in the SZ could be due to the ability of *A. lilaceps* to decompose primarily grass root residue or humic substances similarly to *M. oreades*, as it grows and migrates through soil. Nitrogen compounds released into the soil ammonium pool in SZ may be taken up by plants, as indicated by the increased growth of western wheatgrass found in this study, or immobilized by the fungus or proliferating microbial populations. In the inner zone of the ring, mycelial senescence and consequent reduced release of N compounds eventually results in lower production of western wheatgrass biomass.

The increased growth of western wheatgrass in the stimulation zone of *A. lilaceps* rings, which we observed, typifying herbaceous stimulation by the fairy ring phenomenon [53, 61], was due to a number of factors. The separation of SZ clustering from IN and OUT observed in PCA suggests

Table 7 Data analysis for western wheatgrass: Western wheatgrass cover (2010 and 2011), and 2011 values for number of stems, number of inflorescences, height, and biomass

	IN	SZ	OUT
Cover (%) 2010	1.1 (2.9)b	39.8 (1.7)a	20.0 (6.4)b
Cover (%) 2011	15.6 (21.0)b	42.2 (21.2)a	6.5 (4.8)b
Number of stems	17.1 (5.0)b	121.2 (30.5)a	19.8 (7.2)b
Number of inflorescences	0.7 (0.5)b	3.1 (1.0)a	0.7 (0.9)b
Height (cm)	28.1 (0.8)b	44.4 (3.3)a	29.4 (1.6)b
Biomass (g)	3.4 (1.1)b	34.6 (7.1)a	3.9 (1.2)b

Significant differences among zones for each dependent variable (Tukey's HSD, $P<0.05$) are indicated by different letters. Comparisons were performed on least square means, but nontransformed averages are presented here (one standard deviation)

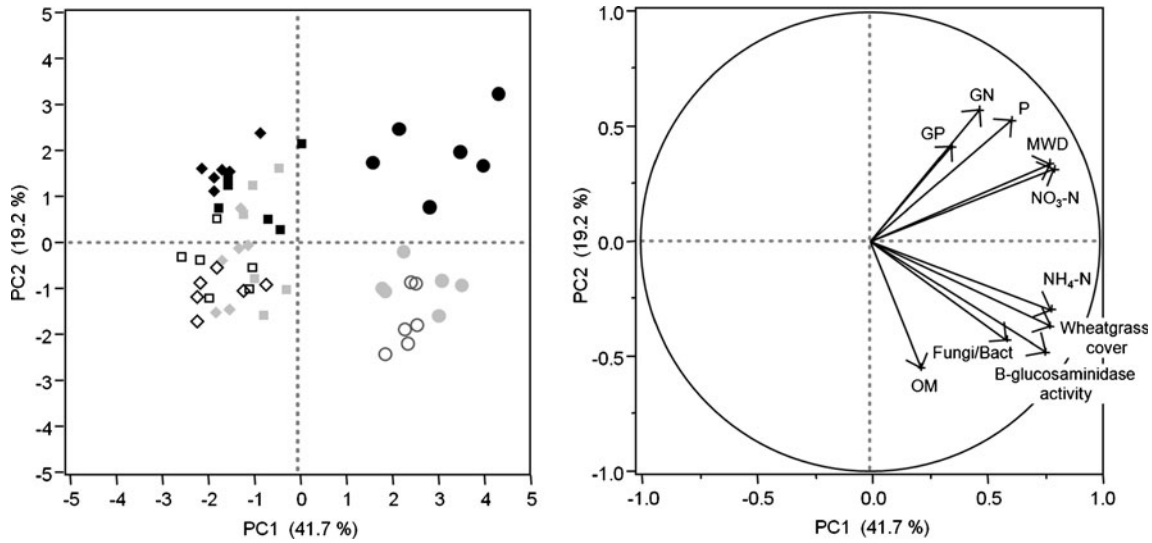

Fig. 3 **a** Principal component analysis based on the correlations of multiple variables [organic matter content, NH_4–N, NO_3–N, and P concentrations, aggregate stability (*MWD*), FAME ratio of Fungi/Bacteria, Gram-positive (*GP*) and Gram-negative (*GN*) bacteria, β-glucosaminidase activity, wheatgrass cover] from each zone. *Circles* SZ; *squares* OUT; *triangles* IN; *solid symbols* location 1; *gray symbols* location 2; *open symbols* location 3. **b** Factor loadings for PCA

that the enhancement of soil macroaggregate formation and stability, which would result in less soil compaction with lower bulk density, and the release of N compounds in the soil pool by the activity of the fairy ring fungus could be some of the factors creating favorable nutritional and environmental soil conditions for western wheatgrass to thrive in SZ. Our data indicated a selective effect of root-adhering soil in SZ towards Gram-negative bacteria, in particular the pseudomonads (members of γ-Proteobacteria), whereas in IN, the selective effect was toward Gram-positive bacteria, in the group of Bacilli-Actinobacteria (Fig. 2). In the OUT zone of the rings, this effect appeared to be less defined than SZ and IN. It has been acknowledged that physical and nutritional factors in soil could influence plant growth and can therefore be presumed to influence both root exudates and rhizospheric microbial populations, particularly the pseudomonad populations [35, 55, 62]. We speculate that the activity of *A. lilaceps* in modifying soil quality (soil aggregation) could indirectly affect the quality/quantity of western wheatgrass root exudates (rhizodeposition) in SZ and therefore could have a selective influence on populations of pseudomonads in root-adhering soil. It is not surprising that the populations of pseudomonads were high in root-adhering soil in SZ since they are fast-growing r-strategists, have copiotrophic characteristics, and compete with other microorganisms for root colonization in nutrient rich conditions [42, 62]. In addition, pseudomonads are well documented to have a beneficial role in plant growth since they secrete siderophores, which decrease iron availability for other microorganisms while improving plant iron assimilation, synthesizing phytohormones, or enhancing mineral uptake by plants [24]. In contrast, the concentric zone inside the ring (IN) indicated a reduction in the amount and activity of the fairy ring fungus, decrease in soil aggregation, and decrease in wheatgrass vegetation cover and plant height when compared to SZ. Thus, it is possible that more Bacilli and Actinobacteria proliferated in the root-adhering soil of wheatgrass in IN since these groups of Gram-positive bacteria can be classified as part of the autochthonous soil microbial communities that have a selective advantage in nutrient-limited soil [25, 37].

In this study, bacterial community assessment was based on isolating bacteria on a low nutrient medium [12, 49] using a spiral plating technique to retrieve the predominant (isolates present at the highest dilution levels) bacteria from soil. This essentially facilitates isolation without suppressing the slow-growing species, thereby achieving a higher recovery of bacteria. We are aware that cultivation-dependent methods to assess microbial communities in soil are in need of improvement since many groups of bacteria have no cultivated representatives. However, using the cultivation-dependent approach, we were able to test the predominant bacterial isolates from root-adhering soil for their function in adhering and binding soil particles, which is difficult to obtain using molecular techniques [48]. Among the predominant culturable bacteria isolated from wheatgrass root-adhering soil, the in vitro soil sedimentation assay indicated that *P. fluorescens* and *S. maltophilia*, members of γ-Proteobacteria, were the most efficient in binding soil in SZ, whereas from IN and OUT, *Bacillus* spp., in particular *B. cereus*, were the most efficient. This suggests that these bacteria may produce extracellular components that act as soil binding agents or they may have adhesive morphological structures on their cell surface. Among the

soil-binding bacteria identified by FAME and confirmed by DNA sequencing (Table 5), many have been investigated for their ability to adhere to various biological and mineral surfaces. Filamentous appendages or pili were demonstrated to be involved in adhesion of such *Bacillus* species as *B. atrophaeus* and *B. cereus* to inert surfaces [26]. Cell surface properties of many *Rhizobium* spp. were investigated for their hydrophobicity and adhesion in sandy soil [40]. Several reports demonstrated that the pseudomonads can adhere to soil particles, roots and nutrient-rich substrates [21, 23]. They can produce extracellular polymer substances, such as gellan gum [29] or alginate [52], which have high adhesive properties. Other predominant species, such as *Stenotrophomonas* spp., which bound soil well in our assays, are documented to possess fimbriae, cell-surface extension involved in biofilm formation due to their strong adhesive properties [31]; they can anchor to mineral surfaces, such as clay particles [44]. Furthermore, *Sphingobacterium* spp. are known to have glycosphingolipids, which possess strong adhesive properties in their cell envelopes [27]. However, many of the soil-aggregating species predominantly found in root-adhering soil of wheatgrass that were strongly soil-binding in vitro (*Sphingobacterium* and *Chryseobacterium* spp.) do not exhibit mucoid colony morphologies in contrast to other species such as *Pseudomonas* spp. Thus, the mechanisms of aggregating soil were apparently not restricted to fluid or mucoid compounds as binding agents.

Fairy rings caused by *A. lilaceps* constitute an example of the pronounced influence a soil fungus can have on western wheatgrass production in grassland. However, the activity of *A. lilaceps* in grassland could occur in less defined and noticeable conditions (not necessarily in rings) resulting in the stimulation of western wheatgrass over large areas. Since western wheatgrass is economically important for summer and winter forage for livestock and ecologically valuable in land reclamation and restoration, an understanding of the mechanisms by which fairy ring fungi influence western wheatgrass growth over large areas could have practical use in agriculture in Eastern Montana and areas of short grass prairie.

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